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Horseradish peroxidase compound I as a tool to investigate reactive protein-cysteine residues: from quantification to kinetics

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ABSTRACT

Proteins containing reactive cysteine residues (protein-Cys) are receiving increased attention as mediators of hydrogen peroxide signaling. These proteins are mainly identified by mining the thiol proteomes of oxidized protein-Cys in cells and tissues. However, it is difficult to determine if oxidation occurs through a direct reaction with hydrogen peroxide or by thiol–disulfide exchange reactions. Kinetic studies with purified proteins provide invaluable information about the reactivity of protein-Cys residues with hydrogen peroxide. Previously, we showed that the characteristic UV–Vis spectrum of horseradish peroxidase compound I, produced from the oxidation of horseradish peroxidase by hydrogen peroxide, is a simple, reliable, and useful tool to determine the second-order rate constant of the reaction of reactive protein-Cys with hydrogen peroxide and peroxynitrite. Here, the method is fully described and extended to quantify reactive protein-Cys residues and micromolar concentrations of hydrogen peroxide. Members of the peroxiredoxin family were selected for the demonstration and validation of this methodology. In particular, we determined the pK_a of the peroxidatic thiol of rPrx6 (5.2) and the second-order rate constant of its reactions with hydrogen peroxide ($(3.4 \pm 0.2) \times 10^7$ M^{-1} s^{-1}) and peroxynitrite ($(3.7 \pm 0.4) \times 10^5$ M^{-1} s^{-1}) at pH 7.4 and 25 °C.

Although hydrogen peroxide has been more frequently associated with pathological and adaptive responses, there are a growing number of studies showing its mediation of normal cell physiology through the reversible oxidation of reactive cysteine (protein-Cys) residues [1–9]. In addition to modifying the activity of enzymes whose catalytic mechanisms rely on reduced Cys residues [10–12], hydrogen peroxide may also modulate protein structure and function by oxidizing allosteric Cys residues [5]. Indeed, numerous proteins and enzymes contain noncatalytic yet highly conserved Cys residues across species, arguing for their role beyond catalysis and metal coordination [5].

Two main mechanisms have been proposed for hydrogen peroxide-mediated signaling through protein-Cys residues [13,14]. Initially, the oxidation status of cellular thiols was thought to be under thermodynamic control, with GSH/GSSG[2] acting as the main cellular redox buffer [14]. Protein-Cys residues and GSSG would dynamically engage in thiol–disulfide exchange reactions and protein-disulfide formation would ultimately be reflected in increased GSSG levels. The realization that direct thiol–disulfide exchange reactions are too slow for signaling [15] led to the kinetically controlled model [13,16]. In this model, hydrogen peroxide signaling is triggered by a few exceptionally reactive protein-Cys targets (sensors) that are preferentially and rapidly oxidized. These sensor proteins confer specificity to the signaling process. Sensors may either directly control biological functions or transduce downstream signals through specific protein–protein interactions and thiol–disulfide exchange reactions leading to eventual protein glutathionylation and the formation of protein disulfides. These posttranslation modifications can alter protein conformation, providing a simple mechanism by which protein-Cys oxidation and reduction modify the structure, interactions, and cellular locations of proteins to modulate cellular processes. Hydrogen peroxide sensors occupy upstream positions in individual signaling pathways, and their identification is of major importance to the understanding of redox signaling. However, accomplishing this task is not trivial.

Abbreviations: BSA, bovine serum albumin; DTPA, diethylenetriamine-*N,N,N′,N″*-pentaacetic acid; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); GSH, glutathione; GSSG, glutathione disulfide; HRP, horseradish peroxidase; HRP-I, horseradish peroxidase compound I; HRP-II, horseradish peroxidase compound II; peroxynitrite, the sum of peroxynitrite anion ($ONOO^-$, oxoperoxonitrate (−1)) and peroxynitrous acid (ONOOH, hydrogen oxoperoxonitrate) unless specified otherwise; PCys, generic protein containing a reactive cysteine residue; Prx, peroxiredoxin.

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The inventory of proteins that sense hydrogen peroxide is principally being compiled by mining thiol proteomes of oxidized protein-Cys residues [17–26]. However, many of the proteins whose Cys residues are found oxidized in cells and tissues by proteomic approaches exhibit moderate reactivity toward hydrogen peroxide in vitro. Notorious examples include protein tyrosine phosphatase 1B [27–29] and glyceraldehyde-3-phospate dehydrogenase [22–25,30]. Some nonreactive protein-Cys residues may be detected as oxidized in cells and tissues because of their high relative abundance. Others may be detected through indirect thiol–disulfide exchange reactions, as implied by the kinetically controlled model. Thus, innovative, simple, and reliable approaches for investigating protein-Cys residues in regard to quantification and reactivity toward hydrogen peroxide are required to determine the upstream sensors of hydrogen peroxide-mediated signaling.

Here, we fully describe the use of horseradish peroxidase (HRP)/hydrogen peroxide as a tool to quantify reactive protein-Cys residues and to determine the second-order rate constants of their reactions with hydrogen peroxide. The method is also useful to determine the pK_a of reactive protein-Cys residues and the second-order rate constant of their reaction with peroxynitrite and to quantify low-micromolar concentrations of hydrogen peroxide.

Principles

Few enzymes have been studied as comprehensively as HRP, an abundant plant heme peroxidase that has been explored in a variety of analytical and technical applications [31,32]. The native enzyme, ferric HRP, uses hydrogen peroxide to oxidize a number of natural and artificial organic compounds to free radicals through the intermediacy of compounds I ($P^{\bullet+}Fe(IV)=O$; HRP-I) and II ($PFe(IV)=O$; HRP-II) (Eqs. (1)–(3)).

$$PFe(III) + H_2O_2 \rightarrow P^{\bullet+}Fe(IV) = O \quad (1)$$

$$P^{\bullet+}Fe(IV) = O + X \rightarrow PFe(IV) = O + X^{\bullet} \quad (2)$$

$$PFe(IV) = O + X \rightarrow PFe(III) + X^{\bullet} \quad (3)$$

HRP is active over a wide pH range, and the second-order rate constant of many its reactions has been determined under a variety of experimental conditions [31,32]. Of particular relevance to the study of reactive protein-Cys residues is the rapid and irreversible oxidation of ferric HRP by hydrogen peroxide ($k=1.7\times10^7\ M^{-1}\ s^{-1}$; pH 7.4; Eq. (1)) [33] and peroxynitrite ($k=1.02\times10^6\ M^{-1}\ s^{-1}$; pH 7.4; [34]) to produce compound I. This oxidation results in a considerable change in light absorption in the visible range ($\Delta\varepsilon_{403nm}=5.4\times10^4\ M^{-1}\ cm^{-1}$), permitting the reaction to be monitored with low concentrations of enzyme and peroxides (low-micromolar range). Additionally, compound I is considerably stable in the absence of reductants, such as X or excess peroxides, which also reduce compound I (Eq. (2)) [31].

The rapid and irreversible formation of HRP-I from HRP and hydrogen peroxide (Eq. (1)) or peroxynitrite coupled with large associated change in light absorption and the stability of HRP-I in the absence of reductants makes this oxidation reaction an excellent analytical tool. Previously, we explored it to determine the second-order rate constant of the reactions of cytosolic yeast peroxiredoxins with peroxynitrite and hydrogen peroxide [35]. Here, we fully describe compound I formation as a tool to determine the kinetic parameters of reactive protein-Cys residues and extend its application to quantify low levels of hydrogen peroxide and to selectively quantify protein-Cys residues highly reactive toward hydrogen peroxide. Members of the peroxiredoxin family (Prx), specifically, rPrdx6 (1-Cys Prx), yTSA1 (typical 2-Cys Prx), and xfBcp (atypical 2-Cys Prx) [35–43], were selected to demonstrate and validate this methodology.

Quantification of hydrogen peroxide with HRP

The rapid reaction of HRP with hydrogen peroxide (Eq. (1)) can be used for the direct, rapid, and sensitive quantification of hydrogen peroxide in solution as illustrated by Eq. (4).

$$(y + x)HRP + x\,H_2O_2 \rightarrow y\,HRP + x\,HRP{-}I + x\,H_2O \quad (4)$$

where $[H_2O_2]_0=[HRP\text{-}I]_\infty=x$

If an unknown but substoichiometric amount of hydrogen peroxide is added to a solution containing excess HRP, it is rapidly and completely consumed, quantitatively yielding HRP-I (Eq. (4)). The amount of hydrogen peroxide added into solution is equal to and can be determined by the quantification of the amount of HRP-I formed. This is easily accomplished by taking the difference in absorbance at 403 nm between an HRP blank (ferric HRP alone) and a sample (ferric HRP + H_2O_2) and dividing by the HRP/HRP-I differential extinction coefficient. Thus, it is a differential assay necessitating that the amount of hydrogen peroxide added is substoichiometric to HRP (see above). The hydrogen peroxide stock solution concentration can be adjusted by serial dilutions to fit this requirement.

Selective quantification of reactive protein-Cys residues

The method we designed for the quantification of reactive protein-Cys residues toward hydrogen peroxide is also a differential assay. Excess hydrogen peroxide ($[H_2O_2]_0$) is mixed with substoichiometric levels of reactive protein-Cys residues and the reaction is left to complete; for highly reactive protein-Cys, such as the peroxidatic Cys of Prx's, complete oxidation will take only a few seconds ($k_{H2O2}\geq10^5\ M^{-1}\ s^{-1}$) [35,44,45]. Then, residual hydrogen peroxide ($[H_2O_2]_\infty$) is quantified as described above, and the reactive protein-Cys concentration is calculated by the difference between the hydrogen peroxide added and that remaining after reaction (Eq. (5)):

$$[\text{Reactive protein}{-}\text{cysteine}] = [H_2O_2]_0 - [H_2O_2]_\infty \quad (5)$$

Kinetics of reactive protein-Cys with hydrogen peroxide and peroxynitrite

Known concentrations of the reactive protein-Cys and ferric HRP and substoichiometric hydrogen peroxide are mixed together, letting both proteins compete for the hydrogen peroxide. After the reaction is complete, the amount of HRP-I formed is determined. The less HRP-I formed, the higher the second-order rate constant of the reaction between reactive protein-Cys and hydrogen peroxide. This process can be put in a quantitative framework (Eq. (6)) and used for the determination of the second-order rate constant. Typically, HRP solutions containing increasing concentrations of the reactive protein-Cys under study are mixed with substoichiometric amounts of hydrogen peroxide or peroxynitrite, and HRP decay is monitored at 398 nm. Then, the percentage of inhibition of HRP oxidation ($F/1-F$) by the reactive protein-Cys is plotted against the concentration of the latter, giving a straight line with a slope equal to the second-order rate constant (Eq. (6)). Although HRP oxidation to HRP-I can be monitored at 403 nm, monitoring it at 398 nm is more reliable for kinetic studies because this wavelength is the isosbestic point between HRP-I and HRP-II. Monitoring light absorption changes at 398 nm precludes that an eventual reduction of HRP-I to HRP-II compromises HRP-I quantification (see, also, Caveats) [46]. Sensitivity is not compromised because the changes in light absorption are similar ($\Delta\varepsilon_{403nm}=5.4\times10^4\ M^{-1}\ cm^{-1}$; $\Delta\varepsilon_{398nm}=4.2\times10^4\ M^{-1}\ cm^{-1}$) [31,46].

$$(F\,/\,1-F)k_{HRP}[HRP] = k_{PCys}[PCys] \quad (6)$$

The accurate determination of k_{PCys} depends critically on knowing the concentration of HRP, hydrogen peroxide, and the reactive protein-Cys residue (Eq. (6)). Thus, quantification of these species is an important step in accessing the kinetic properties of the reaction of reactive protein-Cys with hydrogen peroxide. As described above, HRP can be used for quantification of both hydrogen peroxide and reactive protein-Cys residue levels.

Materials

The following reagents were obtained from the specified commercial sources: horseradish peroxidase (HRP type VI-A; Sigma–Aldrich, P8375), bovine serum albumin (BSA; Sigma–Aldrich, PA7906), hydrogen peroxide (Merck), PD-10 gel filtration column (GE Healthcare, 17-0851-01), sodium dithionite (Sigma–Aldrich, 28–2925), DTPA (Sigma–Aldrich, D1133), sodium dodecyl sulfate (SDS; Sigma–Aldrich, L3771), urea (Sigma–Aldrich, U6504), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (Hepes; Sigma–Aldrich, H3375). The buffers used were all 20 mM Hepes, pH 7.4, either alone or with 100 μM DTPA; 100 μM DTPA and 1% SDS; or 100 μM DTPA and 8 M urea. All solutions were prepared with purified Milli-Q water (Millipore), and all buffers were treated with Chelex-100 to remove metal ion traces.

Expression, site-directed mutagenesis, purification, reduction, and quantification of peroxiredoxins and protein thiols

His-tagged Prdx6 from rat (cloned into pET15b *Nde*I and *Xho*I restriction sites) was expressed in the *Escherichia coli* strain origami (DE3) (Novagen) and purified from cell extracts through cobalt affinity chromatography using the Talon Superflow system 6 (Clontech) with the following protocol: equilibration with buffer A (50 mM Tris–HCl, pH 7.4, 100 mM NaCl) with 1 mM phenylmethylsulfonyl fluoride and wash with 10 column volumes of buffer A with 20 mM imidazole, followed by 2 column volumes of buffer A with 100 mM imidazole, and elution with buffer A with 200 mM imidazole. Subsequently, excess imidazole was removed using a PD10 gel filtration column (GE Healthcare) in 20 mM Hepes, pH 7.4.

Xylella fastidiosa Ohr was expressed and purified as described elsewhere [47]. His-tagged *Saccharomyces cerevisiae* Tsa1 was expressed in the *E. coli* strain Bl 21 (DE3) (Novagen) and purified from cell extracts as previously described [37]. His-tagged Bcp and BcpC47S from *X. fastidiosa* were expressed in the *E. coli* strain (DE3) and purified as previously described [38]. Before the experiments, Prx proteins were reduced with 100-fold excess dithiothreitol (DTT) for 60 min at room temperature and commercial BSA was reduced overnight to render an overreduced BSA sample (denatured BSA; dBSA) [48]. BSA possesses one nonreactive protein Cys residue and 17 disulfide bonds, and its overreduction leads to dBSA whose protein Cys content varies from 7 to 19 [48]. Excess DTT was removed by PD10 gel filtration. Protein concentrations were determined photometrically using the following extinction coefficients at 280 nm calculated using the ProtParam tool (ExPASy Proteomics Server, the Swiss Institute of Bioinformatics, Geneva, Switzerland) available at the Website http://bo.expasy.org/tools/protparam.html: BSA, $\varepsilon_{280} = 4.36 \times 10^4\ M^{-1}\ cm^{-1}$; rPrdx6, $\varepsilon_{280} = 2.556 \times 10^4\ M^{-1}\ cm^{-1}$; xfOhr, $\varepsilon_{280} = 4.595 \times 10^3\ M^{-1}\ cm^{-1}$; yTSA1, $\varepsilon_{280} = 2.35 \times 10^4\ M^{-1}\ cm^{-1}$; and xfBcp and xfBcp C47S, $\varepsilon_{280} = 1.696 \times 10^4\ M^{-1}\ cm^{-1}$. Protein-Cys content was determined by the DTNB assay as previously described [9]. One percent SDS was included to guarantee access of DTNB to buried thiols unless otherwise specified.

Instrumentation

Any commercial UV–Vis spectrophotometer can be used. Plate readers can also be employed but the possibility of scanning the UV–Vis spectrum is recommended to guarantee reliability.

Protocols and applications

Quantification of hydrogen peroxide by the HRP/HRP-I differential assay

Protocol A

(1) Make an HRP stock solution of 1 mM and keep it on ice. We usually make 100 μl.
(2) Make a stock solution of hydrogen peroxide at approximately 2.5 mM in water by serial dilutions of commercial hydrogen peroxide and keep it on ice and protected from light.
(3) Add 8 μl of the HRP stock into 992 μl of buffer in a 1-cm-long quartz cuvette to make the final concentration 8.0 μM (this concentration is just a suggestion). Ferric HRP absorbs strongly at 403 nm ($\varepsilon = 1.02 \times 10^5\ M^{-1}\ cm^{-1}$), so to keep absorbance below 1.0, the maximum concentration of HRP that can be used is approximately 10.0 μM. Because this is a differential method, the concentration of HRP sets the upper limit of the amount of hydrogen peroxide that can be quantified. Record the UV–Vis spectrum between 700 and 300 nm, and read the absorbance at 403 nm (A_0) (Fig. 1A, black trace).
(4) Carefully add 2 μl of the hydrogen peroxide stock solution (step 2) and mix; this will make the final concentration of hydrogen peroxide approximately 5.0 μM (this is also a suggestion, but the final concentration of hydrogen peroxide cannot exceed the HRP concentration). Record the UV–Vis spectrum between 700 and 300 nm and read the absorbance at 403 nm (A_F) within a few seconds to 1 min after the addition of hydrogen peroxide (Fig. 1A, red traces).
(5) Subtract A_0 from A_F. Calculate the concentration of hydrogen peroxide added ($[H_2O_2]_{added}$) into the solution by dividing ($A_0 - A_F$) by the HRP/HRP-I differential extinction coefficient ($5.4 \times 10^4\ M^{-1}\ cm^{-1}$). Calculate the concentration of hydrogen peroxide stock solution ($[H_2O_2]_{stock}$) using Eq. (7).

$$[H_2O_2]_{stock} = (1002 \times [H_2O_2]_{added}) / 2 \quad (7)$$

Application

For a 4.0 μM hydrogen peroxide solution (as ascertained by the nominal supplier concentration), the HRP method gave a mean and standard deviation (SD) of 4.2 ± 0.1 μM ($n = 6$; Fig. 1A) and showed good linearity within the range of 1.0–7.0 μM hydrogen peroxide (Fig. 1B; intercept at 0.0052 μM, $R^2 = 0.992$, $n = 6$) when using 8.7 μM HRP. SD varies little within this range, thus 1.0 μM (10 × SD) is a safe limit of quantification of hydrogen peroxide. The method is more sensitive and reproducible than the widely used photometric measurement of hydrogen peroxide at 240 nm ($\varepsilon_{240} = 43.6\ M^{-1}\ cm^{-1}$), which

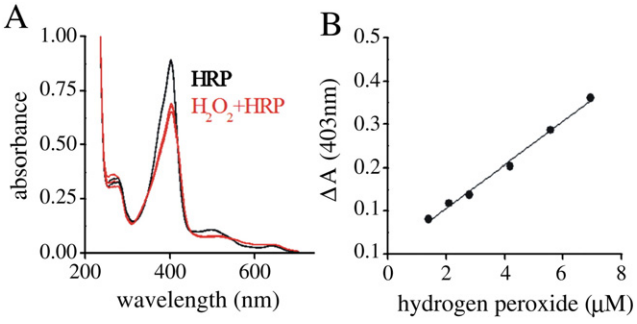

Fig. 1. Differential method for hydrogen peroxide quantification with HRP. (A) HRP alone (8.7 μM, 1 ml in a 1-cm cuvette, black) and substoichiometric hydrogen peroxide added to 8.7 μM HRP in three independent experiments (red). The spectrum acquisition was initiated 1 min after hydrogen peroxide addition (mean ± SD 4.2 ± 0.1 μM, $n = 6$). (B) The assay is linear between 1 and 7 μM hydrogen peroxide ($R^2 = 0.992$, $n = 6$). Incubations were performed in 20 mM Hepes, pH 7.0, 100 μM DTPA at 25 °C.

requires concentrations of hydrogen peroxide in the millimolar range for 1-cm cuvettes. Although the HRP/HRP-I method is less sensitive than the HRP/Amplex red-coupled assay, whose product fluoresces and may be detected in complex biological media [49], the HRP/HRP-I method does not have the intrinsic problems of fluorimetric quantification and does not require a calibration curve.

Selective quantification of reactive protein-Cys residues toward hydrogen peroxide

Protocol B

(1) Make a 1.0 mM HRP stock solution and keep on ice. We usually make 100 µl.
(2) Make a 1.0 mM stock solution of hydrogen peroxide by serial dilutions from commercially available hydrogen peroxide using the supplier nominal concentration. Determine the actual concentration of hydrogen peroxide stock solution as described above (Protocol A, steps 1 through 5). Dilute the stock solution to 600 µM.
(3) Reduce the reactive protein-Cys stock solution with a large excess of any thiol-reducing agent, such as DTT. Use a desalting column to remove excess DTT. Determine the reduced protein-Cys stock solution concentration photometrically (using its extinction coefficient at 280 nm) or any other protein assay. Keep on ice.
(4) Take 10 µl of the HRP stock and add to 990 µl of buffer in a 1-cm cuvette. This makes the final concentration of HRP about 10 µM (this concentration is only a suggestion). Record the UV–Vis spectrum between 700 and 300 nm and read the absorbance at 403 nm (A_0) (Fig. 2A, black trace). Confirm the HRP concentration photometrically ($\varepsilon_{403} = 1.02 \times 10^5\ M^{-1}\ cm^{-1}$); in the experiments of Fig. 2 the actual HRP concentration was 8.7 µM. This control is the hydrogen peroxide blank because there is no oxidation of HRP and the HRP absorbance at 403 nm is maximal.
(5) Add 10 µl of the hydrogen peroxide stock solution (600 µM; Protocol B, step 2) into 980 µl of buffer. Then, add 10 µl HRP stock solution. Homogenize and immediately record the UV–Vis spectrum between 700 and 300 nm (Fig. 2A, red trace). Read the absorbance at 403 nm (A_F). This control is the protein-Cys blank. All of the hydrogen peroxide added reacts with HRP, so oxidation of HRP will be at a maximum and absorbance at 403 nm will at a minimum.

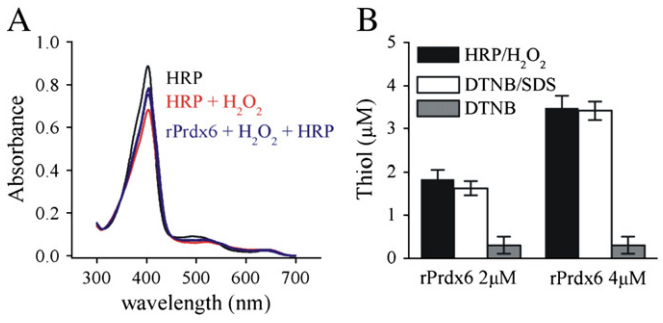

Fig. 2. Quantification of the peroxidatic Cys of rPrdx6. (A) HRP alone (8.7 µM, black trace), 4.0 µM hydrogen peroxide followed by the addition of 8.7 µM HRP (red), and 2.0 µM rPrdx6 (photometric determination) mixed with 4.0 µM hydrogen peroxide and, 10 s later, 8.7 µM HRP; the spectrum was scanned immediately thereafter (blue, three independent experiments). (B) Validation of the HRP differential method against the DTNB method (in the absence and presence of 1% SDS) using rPrdx6, a 1-Cys Prx that possesses only a peroxidatic Cys. The mean values of each method were not statistically different at the 95% confidence interval with different concentrations of rPrdx6. For [rPrdx6] 2.0 µM, HRP method mean = 1.8 ± 0.3 µM and DTNB method mean = 1.6 ± 0.2 µM ($n = 3$); for [rPrdx6] 4.0 µM, HRP method mean = 3.5 ± 0.3 µM and DTNB method mean = 3.4 ± 0.2 µM ($n = 3$). In these experiments, 6.0 µM hydrogen peroxide was employed. Incubations were performed in 20 mM Hepes, pH 7.0, 100 µM DTPA at 25 °C; the DTNB concentration was 100 µM.

(6) Add 10 µl of the hydrogen peroxide stock solution (600 µM; Protocol B, step 2) to 800 µl buffer and then add a volume of the protein-Cys resulting in a nominal concentration of around 5 µM (this concentration is only a suggestion). Homogenize and add sufficient buffer to complete the volume to 990 µl. Wait from a few seconds to 1 min for the reaction between hydrogen peroxide and reactive protein-Cys to complete and then add 10 µl HRP from stock. Homogenize and initiate UV–Vis spectrum acquisition within a few seconds to 1 min (Fig. 2A, blue traces). Read the absorbance at 403 nm (A_{PCys}), which must necessarily lie between A_0 and A_F.
(7) The reactive protein-Cys residue concentration is equal to the added concentration of hydrogen peroxide ($A_0 - A_F/5.4 \times 10^4\ M^{-1}\ cm^{-1}$) minus the hydrogen peroxide concentration remaining after reaction with the reactive protein-Cys ($A_0 - A_{SH}/5.4 \times 10^4\ M^{-1}\ cm^{-1}$) and can be calculated by dividing ($A_{SH} - A_F$) by ($5.4 \times 10^4\ M^{-1}\ cm^{-1}$).

Validation and application

The method was validated with members of the Prx family. These proteins are efficient and versatile Cys-based peroxidases that reduce hydrogen peroxide, peroxynitrite, and a wide range of alkylhydroperoxides [35,44,45] at the expense of thiols such as thioredoxin. These proteins may also be central in hydrogen peroxide-mediated signaling, as Prx members are frequently detected oxidized in thiol proteomes [2,10,22,24–26,30,50,51]. The most remarkable chemical characteristic of Prx is its peroxidatic cysteine residue, which is extremely reactive toward hydrogen peroxide (10^5–$10^7\ M^{-1}\ s^{-1}$). Some members of the Prx family possess resolving Cys and structural Cys residues in addition to the peroxidatic Cys, but only the last reacts rapidly with hydrogen peroxide. Thus, the peroxidatic Cys of Prx is the ideal substrate to test and validate our method.

The stoichiometry of the reaction between the peroxidatic Cys and hydrogen peroxide is 1 to 1, regardless of the Prx class and the catalytic mechanism. Thus, the peroxidatic Cys concentration is equal to the added concentration of hydrogen peroxide minus the hydrogen peroxide concentration remaining after reaction with Prx and can be calculated by dividing ($A_{SH} - A_F$) by ($5.4 \times 10^4\ M^{-1}\ cm^{-1}$) (see above). Fig. 2A illustrates a typical experiment performed to quantitate the peroxidatic and only Cys residue of rPrdx6, a 1-Cys Prx. Two important controls are also shown: the hydrogen peroxide blank (black trace), which corresponds to 8.7 µM ferric HRP, and the Prx blank (red trace), which corresponds to 8.7 µM ferric HRP plus 4.0 µM hydrogen peroxide solution. In this particular case, 4.0 µM HRP is oxidized to HRP-I, leaving 4.7 µM ferric HRP unaltered. These two controls set upper and lower absorbance limits, corresponding to no oxidation and maximal oxidation of HRP with the amount of hydrogen peroxide used in the assay. Sample absorbance must lie within these limits. Quantification of peroxidatic Cys was performed with two different concentrations of rPrdx-6, 2.0 and 4.0 µM (as photometrically measured at 280 nm), yielding values of 1.8 ± 0.2 and 3.5 ± 0.3 µM, respectively. These results were not statistically different from the values determined by the DTNB method (Fig. 2B), thus validating the assay. The SD for peroxidatic Cys quantification was 0.2 µM and thus the safe limit of quantification is 2 µM.

The selectivity of the method for reactive protein-Cys residues was proved by comparing the quantification of protein-Cys residues of various proteins by the HRP/hydrogen peroxide method and the DTNB method (Table 1). As expected from the poor reactivity of nonreactive protein-Cys toward hydrogen peroxide, overreduced BSA (dBSA) [48] and xfOhr [52] are both undetectable by the HRP assay. This assay responds quantitatively to the peroxidatic Cys of rPrdx-6 (a 1-Cys Prx) and does not detect the resolving Cys of yTSA1 (a typical 2-Cys Prx). In contrast, the DTNB assay detects both the peroxidatic and the resolving Cys residues. This is more clearly shown for xfBcp, an atypical 2-Cys Prx that contains two additional Cys residues [38]. All

four Cys residues are reactive toward DTNB in the presence of SDS, but only one is responsive to the HRP assay. Additionally, the assay was not responsive to xfBcp C47S, whose peroxidatic Cys is mutated to Ser, demonstrating the specific recognition of the peroxidatic Cys in the wild-type enzyme (Table 1).

The characteristics of the HRP/hydrogen peroxide method should be emphasized. It eliminates the need to use denaturing agents that are required for quantification with DTNB (compare white and gray bars in Fig. 2B), which is time-consuming and may introduce artifacts into the system. In fact, the HRP/hydrogen peroxide method should be performed under native conditions because it depends on the full activity and reactivity of the reactive protein-Cys residue. An assumption of the method is that there is no significant consumption of hydrogen peroxide by the nonreactive protein-Cys residues, such as resolving and structural Cys. This assumption is reasonable because peroxidatic Cys residues react with hydrogen peroxide 1,000,000- to 10,000,000-fold faster than nonreactive Cys [13], an enormous kinetic resolution that provides a time window more than sufficient for the quantification of residual hydrogen peroxide. The assay is rapid and is performed with low-micromolar levels of hydrogen peroxide and proteins, so reactions of nonreactive protein-Cys and protein-sulfenic acid derivatives (protein-CysSOH) with the remaining hydrogen peroxide are slow enough [53] to be neglected in the time scale of the experiments. We estimate that protein-Cys residues reacting with hydrogen peroxide with rate constants $\geq 10^5\ M^{-1}\ s^{-1}$ (a kinetic resolution of at least a 1000-fold over nonreactive Cys) can be safely quantified. Thus, Ohr, which reacts with hydrogen peroxide with a rate constant of $1\times10^2\ M^{-1}\ s^{-1}$ [52], is virtually undetectable (Table 1).

The main advantage of the HRP/hydrogen peroxide assay in comparison with existing assays is its selectivity to reactive protein-Cys residues. The determination of second-order rate constants is critically dependent on the reactive protein-Cys concentration [40,43], yet this parameter cannot be determined accurately by the DTNB/SDS assay. Indeed, it is usually assumed that thiol recovery is quantitative in the latter assay, but this is rarely the case. Such an assumption causes errors that will generate uncertainties in the kinetic parameters eventually determined for reactive protein-Cys residues.

Table 1
Selective quantification of peroxidatic cysteines by the HRP/hydrogen peroxide method.

Protein	[Protein][a] ([expected thiol]) (μM)	HRP/H_2O_2 (peroxidatic cysteine) (μM)	DTNB (total thiol) (μM)
Nonperoxiredoxins			
dBSA	**1.0 (35.0)**[b]	**ND**[c]	**8.1 ± 0.2**
dBSA	**2.0 (70.0)**	**ND**	**16.8 ± 0.1**
xfOhr	**4.0 (8.0)**	**ND**	**3.4 ± 0.1**
xfOhr	**2.0 (4.0)**	**ND**	**1.7 ± 0.2**
Peroxiredoxins			
rPrdx6	2.0 (2.0)	1.8 ± 0.2	1.6 ± 0.2
rPrdx6	4.0 (4.0)	3.5 ± 0.3	3.4 ± 0.2
yTSA1	2.4 (4.8)	2.3 ± 0.2	4.1 ± 0.2
yTSA1	4.8 (9.6)	4.6 ± 0.2	8.4 ± 0.3
xfBcp	2.0 (8.0)	1.6 ± 0.2	6.6 ± 0.4[d]
xfBcp	4.0 (16.0)	3.7 ± 0.3	14.2 ± 0.3[d]
xfBcp C47S	**2.0 (6.0)**	**ND**	**5.2 ± 0.3**[d]
xfBcp C47S	**4.0 (12.0)**	**ND**	**9.7 ± 0.3**[d]

Boldface denotes proteins with cysteine residues whose reactivities toward hydrogen peroxide are too slow to be detected by the HRP/H_2O_2 method.

[a] As measured photometrically at 280 nm using the respective extinction coefficient and number of cysteine residues in the primary structure: BSA ($\varepsilon_{280} = 4.36\times10^4\ M^{-1}\ cm^{-1}$), rPrdx6 ($\varepsilon_{280} = 2.556\times10^4\ M^{-1}\ cm^{-1}$), xfOhr ($\varepsilon_{280} = 4.595\times10^3\ M^{-1}\ cm^{-1}$), yTSA1 ($\varepsilon_{280} = 2.35\times10^4\ M^{-1}\ cm^{-1}$), xfBcp and xfBcp C47S ($\varepsilon_{280} = 1.696\times10^4\ M^{-1}\ cm^{-1}$).

[b] This number corresponds to the total protein Cys residues of BSA (1 Cys and 17 disulfide bonds) but only part of them is reduced even after long incubations with a high excess of DTT [48].

[c] ND, not detectable.

[d] In the presence of 8.0 M urea.

Kinetics of reactive protein-Cys residues with hydrogen peroxide and peroxynitrite

Protocol C

(1) Hydrogen peroxide and reactive protein-Cys residues are determined as described above in Protocols A and B, respectively.
(2) Prepare five or six solutions containing the same concentration of HRP in the absence and presence of increasing concentrations of the reactive protein-Cys under study and adjust the volume of the solution to 1 ml.
(3) Add a substoichiometric amount of hydrogen peroxide to the above solutions. Within 10 s to 1 min after hydrogen peroxide addition, record the spectrum between 700 and 300 nm (Fig. 3A). To be consistent, initiate the acquisition of the spectrum of all controls and samples at the same time interval after mixing. Read the absorbance at 398 nm (isosbestic point of HRP-I and HRP-II) for each sample and control. The extent of oxidation of HRP should decrease with increasing thiol protein (Fig. 3B).
(4) Plot $(F/1-F)k_{HRP}$ [HRP] vs [PCys] (Eq. (6)) (Fig. 3C) and determine the rate constant for the reaction of Prx with hydrogen peroxide by the slope of the plot. The same steps can be followed to determine the second-order reaction of protein-Cys with peroxynitrite [35,46].

Application

This method was previously validated by showing that it provides the same value of second-order rate constant as stopped-flow kinetics in cases in which the peroxide is peroxynitrite, the only peroxide possessing a distinguishable light absorption in the UV range [38]. To illustrate other applications of the method, we have determined the second-order rate constants of the reactions of rPrdx6 with hydrogen

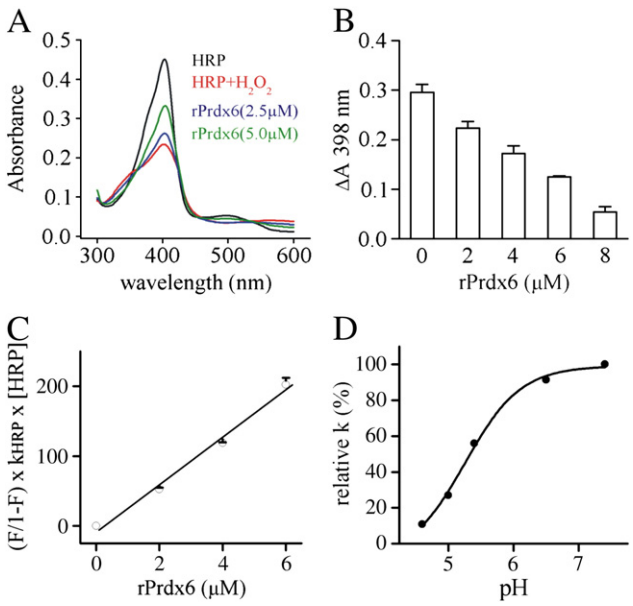

Fig. 3. Determination of the second-order rate constant of the reaction between hydrogen peroxide and rPrdx6 and the pK_a of its peroxidatic Cys residue. (A) Prevention of HRP oxidation by increasing concentrations of rPrdx6. (B) Quantitative inhibition of HRP-I formation by increasing concentrations of rPrdx6. (C) Linear plot of $(F/1-F)k_{HRP}$ [HRP] vs [rPrdx6] (slope $= k_{rPrdx6} = (3.4\pm0.2)\times10^7\ M^{-1}\ s^{-1}$). (D) Variation of the second-order rate constant of the reaction between rPrdx6 and hydrogen peroxide with pH. In (A), (B), and (C) incubations were performed in 20 mM Hepes containing 100 μM DTPA, pH 7.4, 25 °C. In (D) the same conditions were used except for the experiments at acidic pH, which employed 20 mM acetate buffer containing 100 μM DTPA.

peroxide (Fig. 3) and peroxynitrite. Representative experiments show that rPrdx6 prevents the oxidation of HRP to HRP-I by hydrogen peroxide in a concentration-dependent manner (Figs. 3A and B). The plot of $(F/1-F)k_{HRP}$ [HRP] vs [rPrdx6] was linear (Fig. 3C), allowing determination of the second-order rate constant of rPrdx6 oxidation by hydrogen peroxide as $(3.4\pm0.2)\times10^7\ M^{-1}\ s^{-1}$ at pH 7.4 and 25 °C. Similar experiments performed with peroxynitrite provided a second-order rate constant value of $(3.7\pm0.4)\times10^5\ M^{-1}\ s^{-1}$ (pH 7.4 and 25 °C) for the reaction between rPrdx6 and peroxynitrite (data not shown). The HRP competitive approach cannot be applied to study the reaction of Prx with organic peroxides because they react with peroxidase enzymes in processes that are relatively slow and complex and produce radical intermediates [54]. In these cases, alternative approaches are required [35,46,55].

The HRP/hydrogen peroxide method can also be used to determine the pK_a values of reactive protein-Cys residues by studying the variation of their second-order rate constants with pH. This is feasible because the reaction of HRP with hydrogen peroxide is pH-independent in the range of 4.5 to 7.5 [31,56]. In the case of rPrdx6, the pK_a of the peroxidatic Cys was calculated as 5.2 (Fig. 3D). A low pK_a may be characteristic of protein-Cys residues prone to oxidation, but low pK_a alone does not explain the rapid reaction of peroxidatic Cys residues with peroxide. Breaking of the peroxide bond requires the assistance of other residues for rapid oxidation [57]. Because rPrdx6 displays high reactivity toward hydrogen peroxide over a considerable pH range, it can function in the cytosol (pH 7.4) and lysosomes (pH <6.0), where it is considered to have a phospholipase A_2 activity [58].

Previously, the catalytic efficiency (k_{cat}/K_M values) of Prdx6 toward hydrogen peroxide was determined to be in the range of $10^6\ M^{-1}\ s^{-1}$ by steady-state kinetics [59]. This value is 1 order of magnitude lower than the value determined here by competitive kinetics. This apparent discrepancy is probably due to the fact that under the experimental conditions employed in the steady-state approach, reduction by the glutathione/Pi-glutathione transferase system may limit Prdx6 turnover. Indeed, it has been proposed before that the reduction of peroxiredoxins is the rate-limiting step of the overall peroxidatic cycle [35,60]. Here, the HRP competitive approach permitted the determination of the second-order rate constant of the reactions of rPrdx6 with hydrogen peroxide and peroxynitrite and the determination of the pK_a of its reactive cysteine residue.

The HRP competitive kinetic approach can be extended to other proteins that react fast with hydrogen peroxide, such as selenium-dependent proteins (mammalian glutathione peroxidases) and transcription factors, such as OxyR [61]. As previously emphasized, many proteins suspected to be involved in hydrogen peroxide-mediated signaling have an undetermined reactivity toward hydrogen peroxide. However, these data would be valuable because reactivity is not easily predicted solely on the basis of microenvironment structural analysis.

Caveats

An inherent problem of the HRP/hydrogen peroxide method is the high reactivity of HRP-I, which undergoes one-electron and two-electron reduction cycles with suitable substrates (Eqs. (1)–(3)) [31,32]. These reactions can potentially complicate data analysis and require some attention. HRP-I is stable for several minutes in the absence of electron donors, including hydrogen peroxide, which, as consequence, should be employed in substoichiometric amounts. Thus, reduction of HRP-I to HRP-II and HRP should not be a problem in quantifying hydrogen peroxide (Fig. 1) and reactive protein-cysteine residues (Fig. 2). In the latter case, a possible source of error is contamination of the protein under study by the agent employed to reduce it, such as DTT. If considerable HRP-II formation is detected by its distinctive UV–Vis spectrum ($\varepsilon_{420nm}=1.05\times10^5\ M^{-1}\ cm^{-1}$) [31], the thiol protein should be repurified for complete removal of the reductant. Also, we recommend the use of fresh HRP stock solutions because a few times we did observe HRP-II formation by unknown mechanisms with aged HRP stock solutions.

It should be noted that, in addition to reacting with reactive protein-Cys, HRP-I may oxidize other solvent-exposed protein residues, such as cysteine, tyrosine, and tryptophan [62]. As shown for BSA and for several Prx proteins (Table 1), this is not a problem on the time scale suggested for the experiments (UV–Vis acquisition within 10 s to 1 min after HRP addition). However, each protein has its particularities and it is impossible to completely exclude the possibility that some solvent-exposed residues of a determined protein will be rapidly oxidized by HRP-I. If this occurs to some extent, it will be revealed by a shift of the maximum of the UV–Vis spectrum to longer wavelengths due to the presence of HRP-II. In these cases, thiol quantification and kinetic experiments should monitor HRP-I formation at 398 nm. This is the isosbestic point between HRP-I and HRP-II and its use precludes that an eventual reduction of HRP-I to HRP-II compromises HRP-I quantification.

Actually, in the case of kinetic studies, we recommend the monitoring of HRP-I formation at 398 nm (Fig. 3). Reduction of HRP-I to HRP-II is more likely to occur in kinetic experiments with peroxynitrite because stock solutions of this oxidant are usually contaminated with nitrite [46,48]. HRP-I oxidizes nitrite with a relatively small second-order rate constant ($6.5\times10^3\ M^{-1}\ s^{-1}$) [63] but the process may become significant if nitrite contamination is high. Previously, using peroxynitrite solutions with low levels of nitrite (<20%) we did not observe HRP-II formation and followed HRP-I at 403 nm [35]. However, to be on the safer side, it is better to follow it at 398 nm.

Both the quantification of reactive protein-Cys residues and the competitive kinetic approach are based on hydrogen peroxide consumption. Consequently, the assays will respond to any protein reactive toward hydrogen peroxide, Cys-based or not. If the goal is the quantification and kinetics of a reactive protein-Cys, identification of an essential Cys is imperative. The method described here requires protein expression and purification and must be performed for each protein individually. Nevertheless, it is reliable and simple and provides invaluable information about proteins containing reactive Cys residues.

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